Mechanisms Behind *Ex vivo* CD39 Cardioprotection

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for gradation *with research distinction* in the undergraduate colleges of The Ohio State University

By
Lauren Goodman

The Ohio State University
June 2011

Project Advisors: Dr. Richard Gumina, Department of Internal Medicine,
Professor Olli Tuovinen, Department of Microbiology

Introduction

Ischemia reperfusion injury (I/R) underlies a plethora of pathologies from spinal cord injury to myocardial infarction (MI). Ischemia is a restriction of blood flow to tissues and is often followed by reperfusion, or the return of blood flow. Ectonucleoside triphosphate diphosphohydrolase 1 (CD39) is an innate cardiovascular, blood, and vasculature protective protein.

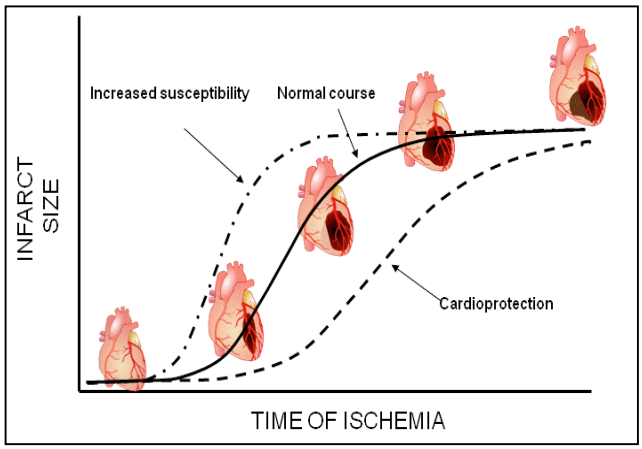


Figure 1. Simplified view of the effect of susceptibility of the heart to I/R injury. Conditions that increase cellular damage render the heart more susceptible to injury while those that decrease myocardial injury convey cardioprotection.

Genetic deletion of CD39 results in loss of the inherent cardioprotection and increases the extent of heart tissue injury after induced MI compared to wild-type mice. As depicted in **Figure 1**, conditions that provide cardioprotection confer a reduced infarct size whereas conditions that increase susceptibility to injury result in a larger infarct. Administration of apyrase, a CD39-like molecule, protects the heart, decreasing damage after MI.[16] However, whether increased CD39 conveys cardioprotection in an isolated heart perfusion, without the complicating factors of blood and hormonal influences has not been determined.

CD39, an endothelial cell-expressed enzyme, rapidly converts ATP and the prothrombotic molecule ADP to AMP.[19] AMP is further degraded by another enzyme, CD73, to adenosine which is anti-thrombotic, vasodilatory, and

cardioprotective.[19] Administered adenosine is cardioprotective in animal models of ischemia-reperfusion injury.[17] Clinical studies have shown that reduced CD39 activity in coronary atherosclerotic lesions predisposes patients to vascular events wherein CD39 is decreased in lesions with unstable angina compared with those with stable angina. [22]

Hypothesis 1: CD39 overexpression mediates cardioprotection, attenuating ischemia/reperfusion (I/R) injury *ex vivo*, decreasing infarct size.

Specific Aim 1: To define the effect of CD39 activity on infarct size following I/R injury.

Anticipated Results: Infarct size will be reduced in CD39TG isolated hearts compared to wildtype.

Hypothesis 2: CD39 overexpression mediates cardioprotection, attenuating ischemia/reperfusion injury, by increasing adenosine levels in the heart.

Specific Aim 2: To define the effect of CD39 activity on ATP, ADP, AMP, and adenosine levels following 30 minutes of ischemia and 15 minutes of reperfusion in isolated hearts.

Anticipated Results: Adenosine levels will be increased in CD39TG isolated hearts compared to wildtype with a consequent decrease in ATP, ADP, and AMP.

Hypothesis 3: CD39 overexpression mediates cardioprotection, attenuating ischemia/reperfusion injury, via the RISK (Reperfusion Injury Salvage Kinase) pathway.

Specific Aim 3: To examine activated levels of AKT, ERK, and deactivation of GSK by examining phosphorylation of these proteins in heart tissue homogenate after *ex vivo* I/R injury.

Anticipated Results: Phosphorylation will be increased in varying degrees in AKT, ERK, and GSK in CD39TG tissue consistent with activation of the RISK pathway.

A mouse model of global overexpression of human CD39 (CD39TG) was utilized in these experiments. The *ex vivo* technique of excising the heart and perfusing it retrograde using a Langendorff perfusion apparatus allows for investigation of

CD39 overexpression only in the heart tissue. After subjecting CD39TG and wildtype hearts to I/R injury, three analyses were conducted to investigate the specific aims described above: 1) infarct size; 2) adenine nucleotide determination by HPLC; 3) RISK protein analysis by Western blotting.

Methods

Langendorff-perfused heart preparation [9,25,26]: Briefly, mice were anesthetized with ketamine (55mg/kg) and xylazine (15mg/kg), and hearts were excised, aortas were cannulated, and hearts were perfused in a Langendorff mode at a constant pressure of 80 mmHg with a modified Krebs-Henseleit buffer (KHB) equilibrated with 95% O_2-5% CO_2 at 37°C (**Figure 2**). The constituents of KHB are (in mM) 120 NaCl, 5.9 KCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.5 EDTA, and 16.7 glucose. A fluid-filled balloon was inserted into the left ventricle (LV) across the mitral valve and connected to a pressure transducer permitting continuous measurement of LV pressure (LVP). Hearts were immersed in a water-jacketed bath maintained at 37°C, and the LV balloon was filled with water to yield a LV diastolic pressure of 3–6 mmHg. Aortic pressure and LV developed pressure (LVDP) was

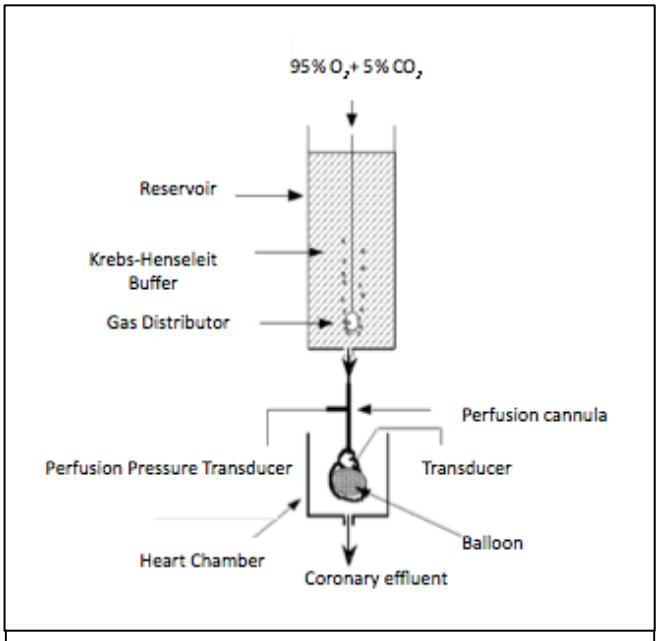


Figure 2. Schematic of a Langendorff heart perfusion setup modified from Sutherland and Hearse website: http://www.southalabama.edu/ishr/help/hearse/

recorded on a PowerLab/400 multichannel data acquisition system (ADInstruments). The LVP signal was digitally processed (using PowerLab Chart software version 4.2; ADInstruments) to yield diastolic and systolic pressures as well as heart rate. Hearts having unexpected arrhythmias during equilibration were excluded from the study. Hearts were subjected to one of three protocols (**Figure 3**). For infarct analysis hearts underwent 30 minutes perfusion (equilibration), 30 minutes ischemia, followed by 60 minutes of reperfusion. For baseline HPLC and Western blot analysis, hearts underwent 60 minutes of equilibration. HPLC and Western blot injured hearts underwent 30 minutes equilibration, 30 minutes of ischemia, followed by 15 minutes of reperfusion. To induce ischemia, perfusion to the heart was stopped for a designated period. Flow of the Krebs-Henseleit buffer was restored for reperfusion.

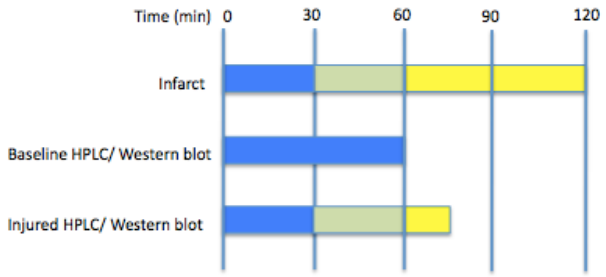


Figure 3. Timeline for Langendorff perfused hearts where blue represents equilibration, green represents ischemia, and yellow represents reperfusion.

Infarct Assessment: Following I/R injury, 1% 2,3,5-triphenyltetrazolium chloride (TTC) was pushed through an aortic cannula to stain viable tissue. (TTC stains viable myocardium red and the area of infarction remains pale). The heart was sectioned and each cross-section was weighed. Infarcts were measured using the computer program MetaVue. Briefly, photographs of cross-sections of the

ventricles were taken under a microscope and the image was transferred into MetaVue. Measurements of the total cross-sectional area, lumen, and infarct were collected and the percent of infarct was calculated. The measurements were repeated for the reverse side of the cross- section for accuracy, and reported as percent infarcted tissue per area at risk. The area at risk and the infarct size were normalized to the sample weight.

Measurement of ATP, ADP, AMP, Adenosine using HPLC: Unless stated all chemicals were obtained from Sigma Aldrich, St. Louis, MO. Mouse hearts were flash frozen with stainless steel mortar and pestel and stored in liquid nitrogen. Samples were fragmented under liquid nitrogen with ceramic mortar and pestel, homogenized with ice cold 0.4 M perchloric acid in a glass Dounces homogenizer for 2 minutes on ice (mg pulverized tissue x 3 = volume perchloric acid). The homogenate was neutralized with 0.4M potassium phosphate dibasic (mg pulverized tissue x 3 = volume dibasic) and incubated on ice for 10 minutes. The homogenate was spun at 16,000g for 5 minutes at 4°C. The supernatant was removed and centrifuged in a 0.5 micron nylon filter at 16,000g for 5 minutes at 4°C. 50 μl of the processed homogenate was loaded into HPLC tubes. Standards were processed in the same manner and utilized to obtain a standard curve (GraphPad Prizm) from which the concentration of the adenine nucleotides, reported in μmol/mg protein, are calculated using linear regression then normalized to protein using the following equation:

[Average conc. Adenine nucleotide (μmole/L)] / [Protein conc. (mg/L)] = conc. μmol/mg protein

Figure 4 provides a spec of the HPLC standards data from which the standard curve was obtained.

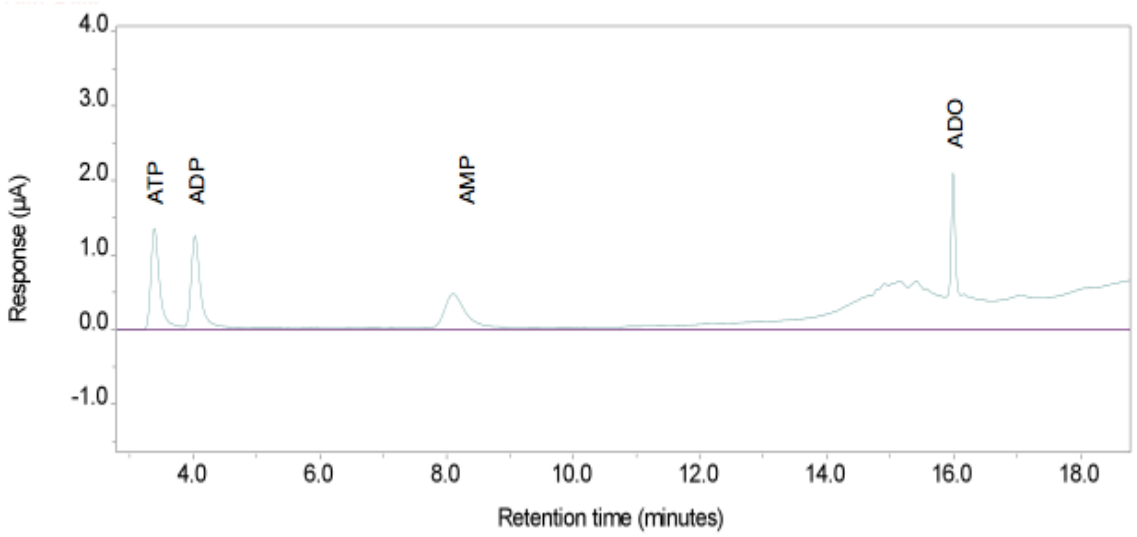


Figure 4. HPLC data obtained for 10μM concentration of ATP, ADP, AMP, and adenosine. 2μM, 5μM, and 10μM standards were obtained to generate standard curves.

Western Blot Analysis: On ice, 400 μl homogenizing buffer and 8 μl protease inhibitor were added to each frozen half of a heart. Samples were homogenized, solubilized for 30 minutes, and centrifuged at 13,000 rpm for 10 minutes. Supernatant was added to glycerol so the final concentration of glycerol is 10%. Aliquots were frozen at -80°C for subsequent analysis. Equal concentrations of protein verified by Coomassie staining were loaded onto SDS PAGE gels and transferred to nitrocellulose membrane overnight at 4°C. Membranes were rinsed and blocked with 5% blocking buffer (5g dried milk, 0.3g NaCl, 95 ml autoclaved

water, and 5 ml 1M Tris pH 7.5) for 2 hours with agitation. Primary antibodies (see **Table 1**) were added in blocking buffer to the membranes and incubated overnight at 4°C. Membranes then were rinsed for one hour with TBS with 0.05% Tween, and anti-rabbit secondary antibodies in 5% blocking buffer were added for 45 minutes (1:5,000) at room temperature. After a one hour rinse with TBS with 0.05% Tween, Supersignal (Pierce) was added to each membrane for five minutes. Kodak Biomax film was then exposed to membranes and developed. Results are quantified using Quantity One software on a BioRad ChemiDoc.

Table 1. Antibodies for Western blot analysis of RISK pathway proteins.

Antibody	Company	Catalog #	Antibody type	Dilution
GAPDH	Cell Signaling	2118	Rabbit Monoclonal	1:5,000
Akt	Cell Signaling	9272	Rabbit Polyclonal	1:5,000
Akt-P-ser	Cell Signaling	9271	Rabbit Polyclonal	1:1,000
GSK	Cell Signaling	9315	Rabbit Monoclonal	1:1,000
GSK-P	Cell Signaling	9322	Rabbit Monoclonal	1:1,000
Erk	Cell Signaling	4695	Rabbit Monoclonal	1:1,000
Erk-P	Cell Signaling	9101	Rabbit Monoclonal	1:1,000

Results

Infarct Analysis

An isolated Langendorff mouse model of myocardial infarction was used to determine the effect of CD39 overexpression without blood and hormonal influences. We measured myocardial infarct size in CD39TG mice compared to

WT mice after 30 minutes ischemia and 60 minutes reperfusion as shown in **Figure 5**. Again, TTC stains viable tissue red, whereas infarcted (dead) tissue will not retain the dye and appears pale. Thus, infarcts were measured as an indication of the extent of cardiovascular injury. The average percent of infarct tissue in WT mice (57 +/- 5%) was significantly greater ($p < 0.0001$) than the average infarct seen in CD39TG mice (21 +/- 2%).

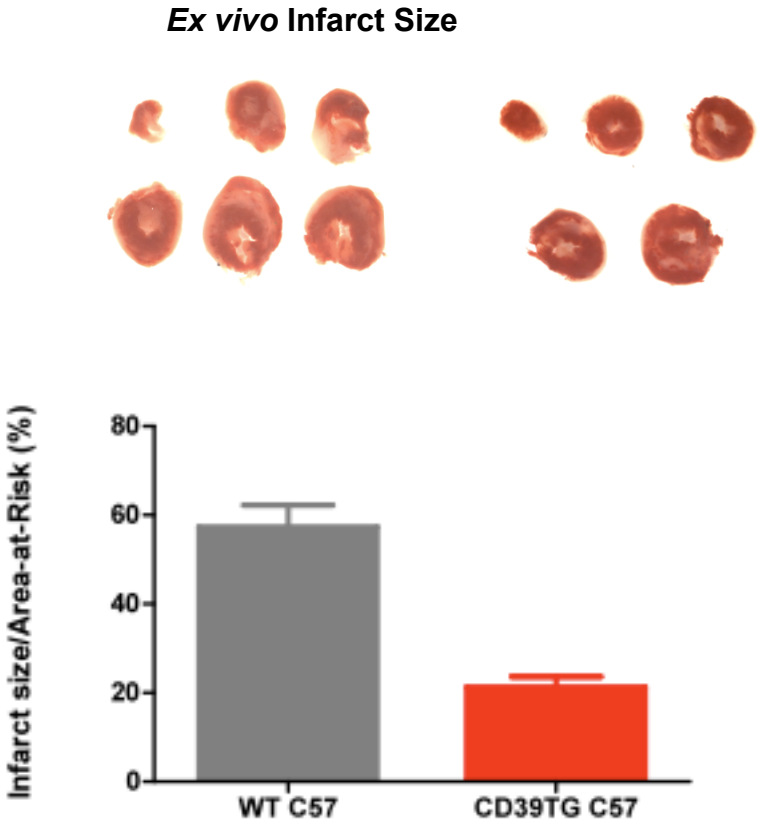


Figure 5. Infarct size seen in WT and CD39TG mice (C57 background) following *ex vivo* 30 minutes ischemia and 60 minutes reperfusion. Above each bar are representative infarct sections. Staining after I/R injury leaves viable tissue red and infarct pale. N = 6/group

Adenine Nucleotide Analysis

To assess the effect of overexpression of CD39, we measured the levels of ATP, ADP, AMP, and adenosine in Langendorff perfused wildtype and transgenic

hearts at baseline and following I/R injury. ATP concentrations measured in μmol/mg protein were taken for WT and CD39TG at both baseline and after 30 minutes ischemia followed by 15 minutes reperfusion. At baseline, the mean concentration of ATP in WT hearts (0.0374 +/- 0.0056 μmol/mg protein) was higher but not significantly different from ATP concentration of CD39TG hearts (0.0182 +/- 0.0079 μmol/mg protein). Following I/R injury the mean concentration of ATP in WT hearts (0.0242 +/- 0.0027 μmol/mg protein) was not significantly higher than ATP levels in CD39TG hearts (0.0177 +/- 0.0031 μmol/mg protein).

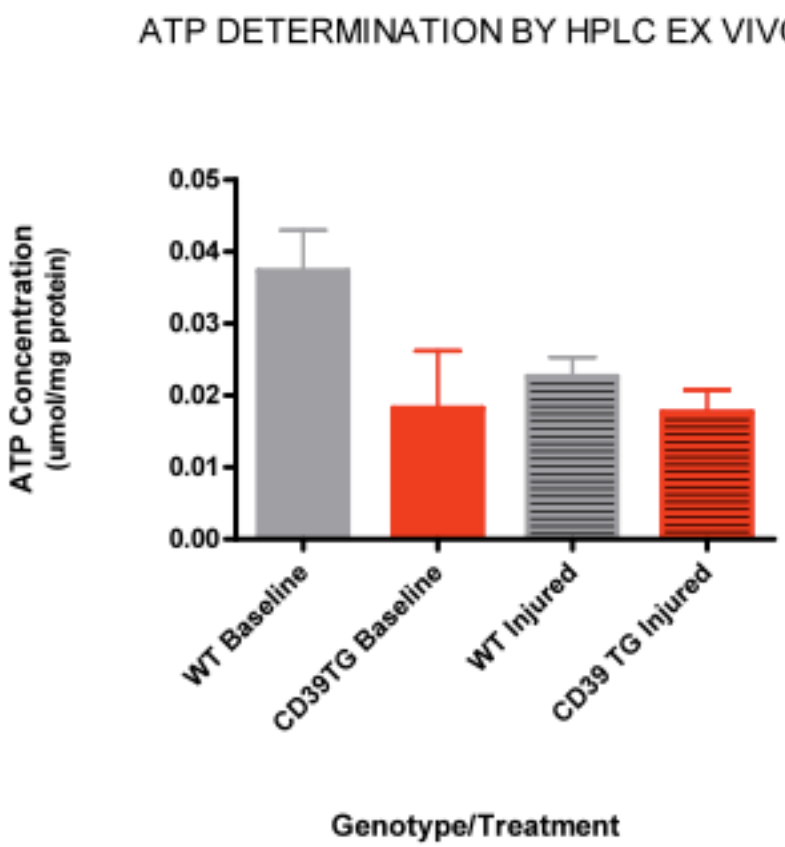


Figure 6.

Figure 6. HPLC measurements of ATP in WT and CD39TG *ex vivo* hearts after 30 minutes ischemia and 15 minutes reperfusion. N=3/group

At baseline, the mean concentration of ADP in WT hearts (0.0473 +/- 0.0091 μmol/mg protein) was greater but not significantly different than in CD39TG

hearts (0.0183 +/- 0.0099 μmol/mg protein). Following I/R injury, the mean concentration of ADP in WT hearts (0.0206 +/- 0.0037 μmol/mg protein) was not significantly different than ADP levels in CD39TG hearts (0.0120 +/- 0.0057 μmol/mg protein). **Figure 7.**

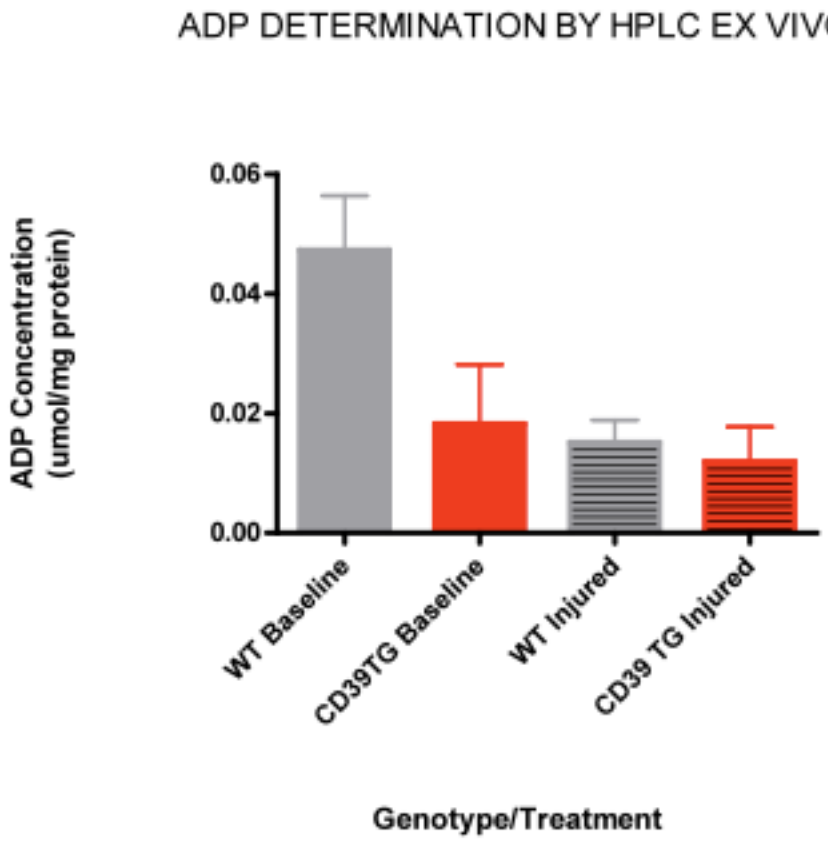


Figure 7. HPLC measurements of ADP in WT and CD39TG *ex vivo* hearts after 30 minutes ischemia and 15 minutes reperfusion. N=3/group

At baseline, the mean concentration of AMP in WT hearts (0.0530 +/- 0.0102 μmol/mg protein) was not significantly different than in CD39TG hearts (0.0252 +/- 0.0143 μmol/mg protein). The mean concentration of AMP in WT after I/R injury (0.0234 +/- 0.0104 μmol/mg protein) was not significantly different than

AMP levels in CD39TG injured mice (0.0223 +/- 0.0086 μmol/mg protein). (p = 0.296). **Figure 8.**

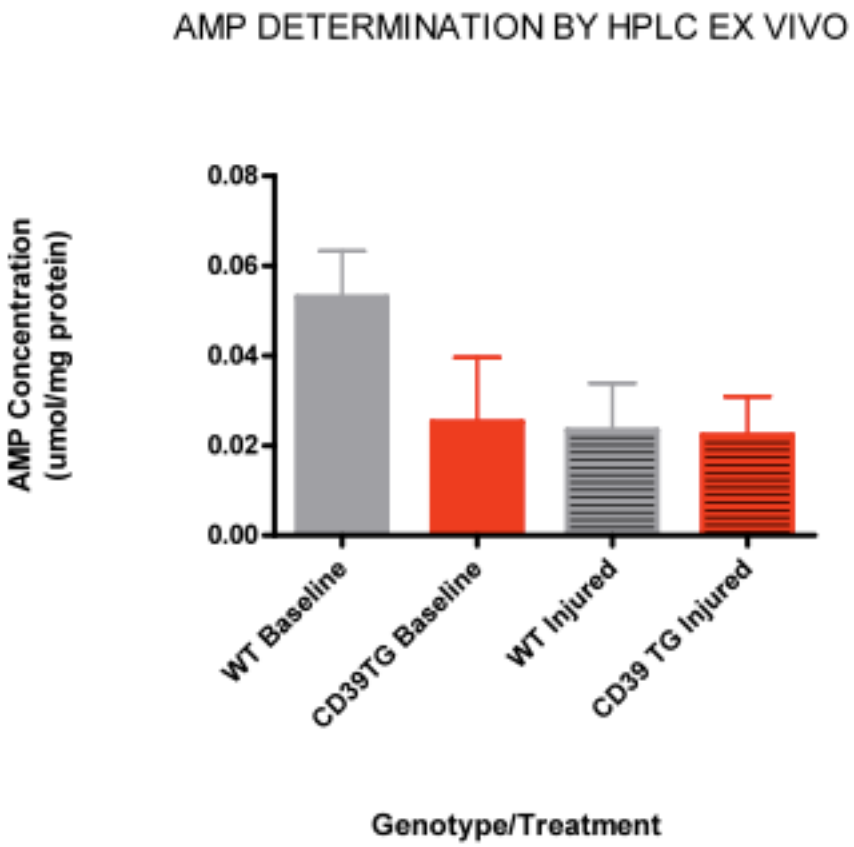


Figure 8. HPLC measurements of AMP in WT and CD39TG *ex vivo* hearts after 30 minutes ischemia and 15 minutes reperfusion. N=3/group

At baseline, the mean concentration of adenosine in WT hearts (0.0059 +/- 0.0012 μmol/mg protein) was not significantly different than adenosine concentrations found in CD39TG hearts (0.0067 +/- 0.0012 μmol/mg protein). However, following I/R injury there was a significant difference in the adenosine concentration in WT hearts (0.0023 +/- 0.0004 μmol/mg protein) verses CD39TG hearts (0.0146 +/- 0.0037 μmol/mg protein, p = 0.005). Indeed, in WT hearts

adenosine decreases, whereas in CD39TG hearts adenosine increases. **Figure 9.**

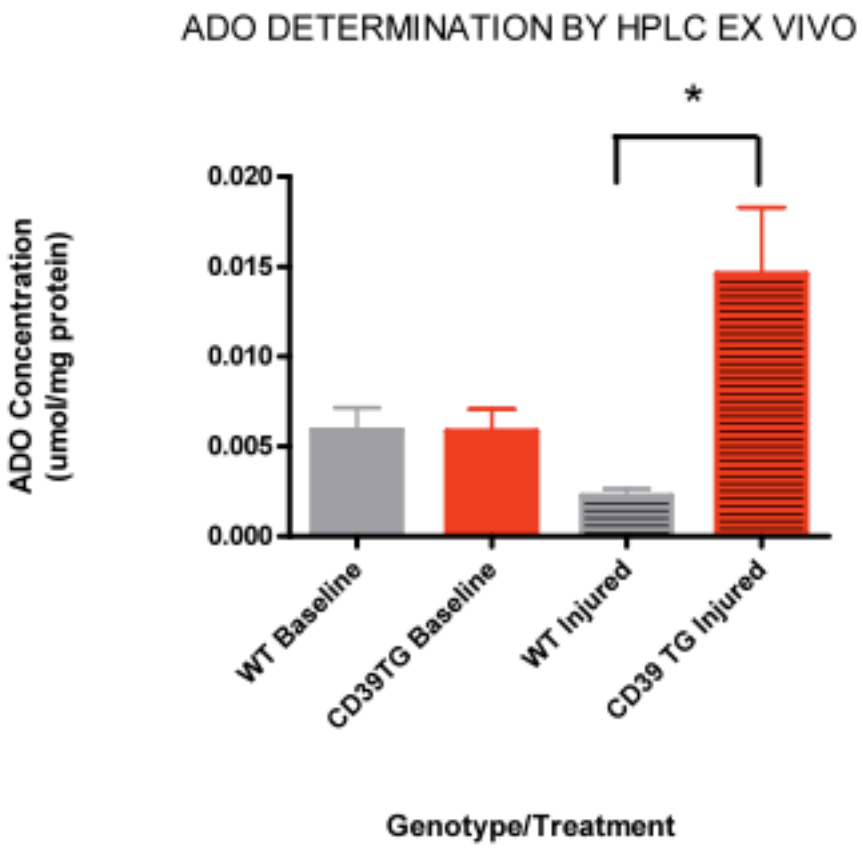


Figure 9. Summary of HPLC measurements of adenosine in WT and CD39TG *ex vivo* hearts after 30 minutes ischemia and 15 minutes reperfusion. N=3/group

RISK Protein Analysis

To begin to decipher the mechanisms behind the cardioprotection seen with CD39 overexpression, Western Blot analysis was performed to examine differences in protein activation between wildtype and transgenic mice at baseline and after 30 minutes ischemia and 15 minutes reperfusion. GAPDH

was used as a control to ensure equal levels of protein were loaded in each sample. There was no statistical significance ($p > 0.05$) between GAPDH concentrations in WT baseline (307.9 +/- 61.1 arbitrary densitometry units), CD39TG baseline (329.4 +/- 38.5 arbitrary densitometry units), Injured WT (326.4 +/- 37.1 arbitrary densitometry units), and injured CD39TG (294.7 +/- 44.3 arbitrary densitometry units). **Figure 10**.

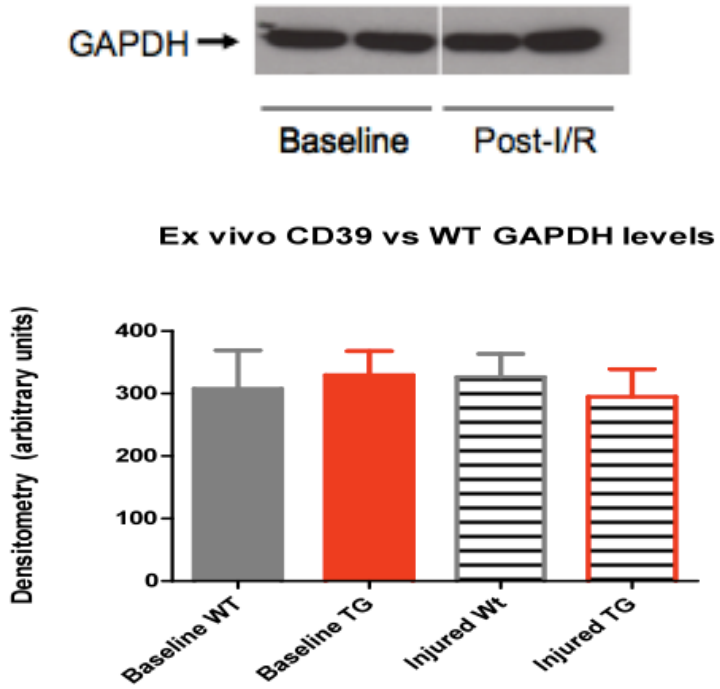


Figure 10. Levels of GAPDH in baseline WT, baseline CD39TG, injured WT, and injured CD39TG show no significant difference in protein loading. N= 4/group, $p > 0.05$.

Given recent publications implicating the RISK pathway in cardioprotection after myocardial infarction, protein kinase B (Akt), Extracellular signal-Related Kinase (Erk), and glycogen synthase kinase (GSK) total protein levels and phosphorylation levels were assessed after 30 minutes ischemia and 15 minutes reperfusion. A time point at 15 minutes reperfusion was used to determine the

early modifications of these proteins. Total Akt protein levels did not change between the experimental groups ($p > 0.05$). However, wildtype hearts demonstrated a significant increase ($p<0.001$) in Akt serine 473 phosphorylation levels (163.9 +/- 13.0 arbitrary densitometry units) after 30 minutes ischemia and 15 minutes reperfusion compared to baseline WT (16.51 +/- 0.88 arbitrary densitometry units). In addition, CD39 transgenic hearts demonstrated an even greater increase ($p<0.001$) in Akt serine 473 phosphorylation levels after injury (Baseline CD39TG: 65.81 +/- 15.71 arbitrary densitometry units, Injured CD39TG: 301.2 +/- 11.7 arbitrary densitometry units, **Figure 11**). There was also a significant difference ($p<0.001$) between injured WT and injured CD39TG showing an increase in phosphorylation and activation of Akt serine in CD39TG mice after 30 minutes ischemia and 15 minutes reperfusion.

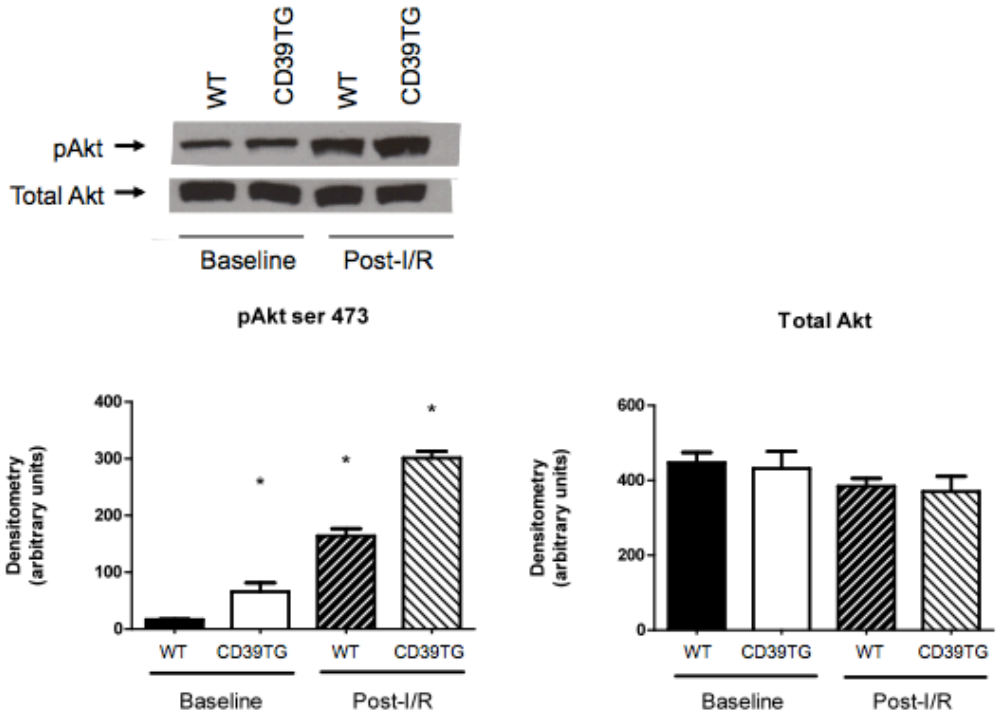
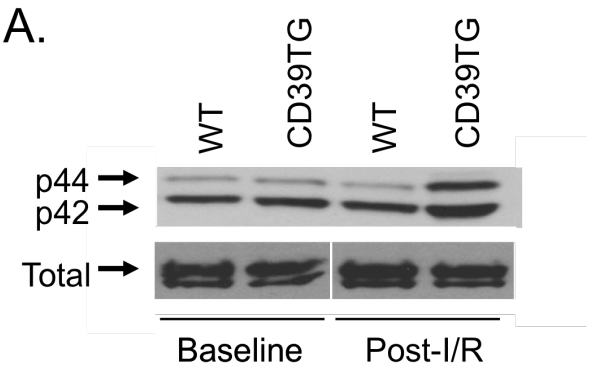
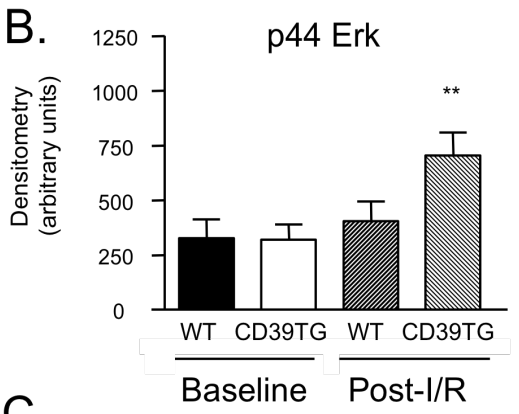
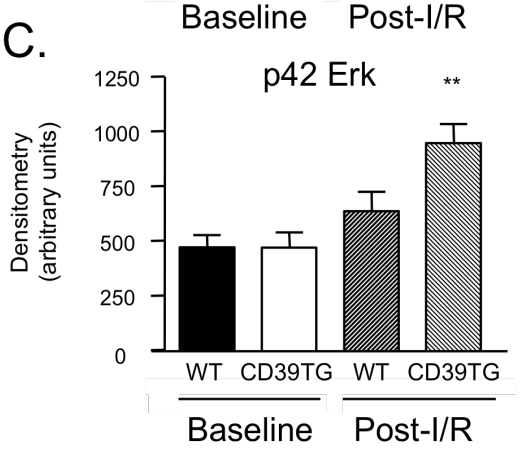
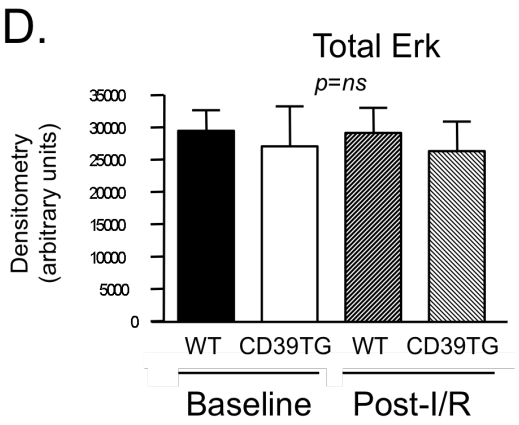


Figure 11. Data from Akt Western blot analysis between *ex vivo* baseline WT, injured WT, baseline CD39 TG, and injured CD39 TG mice. N = 4/group









Although there was an increase in phosphorylation of Erk 42 in WT injured compared to baseline, the difference was not significant, (Baseline WT 471.1 +/- 56.2 arbitrary densitometry units, Injured WT 636.2 +/- 87.8 arbitrary densitometry units; $p > 0.05$). Erk 42 phosphorylation was significantly increased in CD39 transgenic hearts after injury compared to baseline transgenic levels (Baseline CD39TG: 470.1 +/- 68.8 arbitrary densitometry units, Injured CD39TG: 947.9 +/- 86.2 arbitrary densitometry units; $p = 0.002$).

Phosphorylation of Erk 44 mimicked that of Erk 42, **Figure 12.** Again, there was an increase in phosphorylation of Erk 44 in WT injured compared to baseline,

Figure 12. Data from Erk Western blot analysis between *ex vivo* baseline WT, injured WT, baseline CD39 TG, and injured CD39 TG mice. N = 4/group

although the difference was not significant (Baseline WT: 326.5 +/- 86.4 arbitrary densitometry units, Injured WT: 404.8 +/- 89.1 arbitrary densitometry units, p > 0.05). Erk 44 phosphorylation was significantly increased in CD39 transgenic isolated hearts after injury compared to baseline transgenic levels (Baseline CD39TG: 319.7 +/- 70.0 arbitrary densitometry units, Injured CD39TG: 703.4 +/- 105.3 arbitrary densitometry units, p = 0.031). Total Erk protein levels did not change between the experimental groups (p > 0.05)

Next, phosphorylation of GSK, which is downstream in the signaling cascade to Akt and Erk 1/2, was examined (**Figure 13**). Total GSK protein levels did not change between the experimental groups (p >0.05). Although GSK phosphorylation did increase after injury in WT isolated hearts after 30 minutes ischemia and 15 minutes reperfusion, there was not a significant increase compared to baseline levels in wildtype littermates (baseline WT: 85.14 +/- 12.42 arbitrary

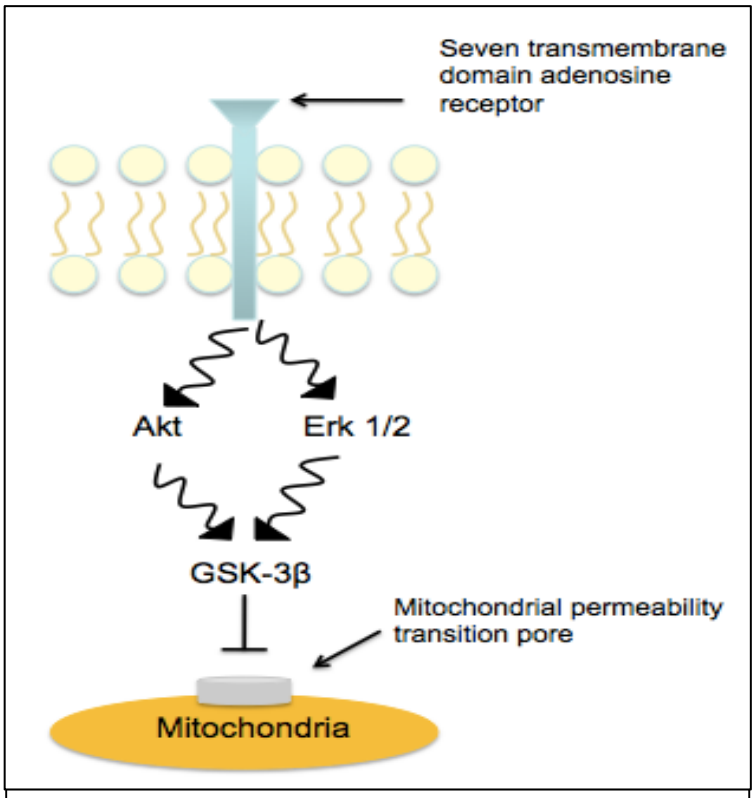
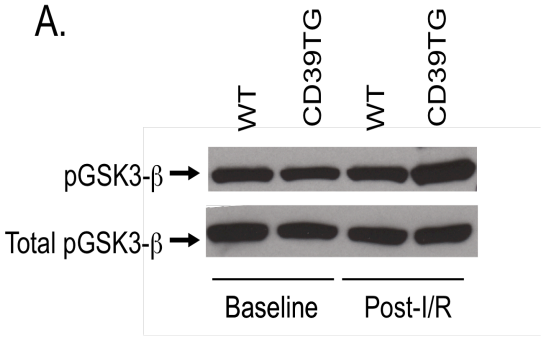
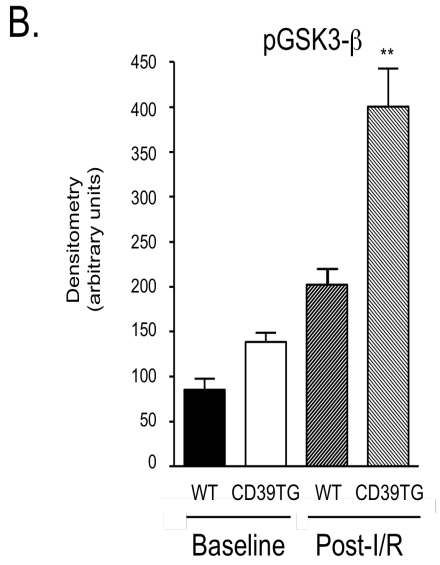
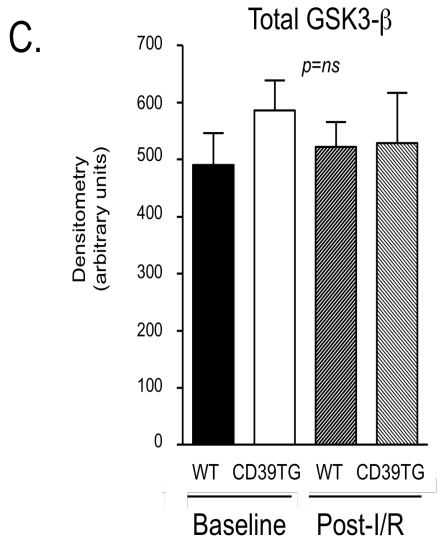


Figure 13. A schematic representation of the adenosine receptor and activation of the RISK pathway. The adenosine receptor is a seven transmembrane domain receptor. Once the receptor binds adenosine, Akt and Erk 1/2 are activated via phosphorylation. Through a signaling cascade, both converge to phosphorylate and deactivate Gsk-3β.







densitometry units, Injured WT: 202.2 +/- 17.7 arbitrary densitometry units; p > 0.05). On the contrary, CD39TG hearts displayed a significant increase in GSK activation after 30 minutes of ischemia and 15 minutes of reperfusion, (baseline CD39TG: 138.5 +/- 10.3 arbitrary densitometry units, injured CD39TG: 342.5 +/- 65.2 arbitrary densitometry units; p = 0.001),

Figure 14.

Figure 14. Data from GSK Western blot analysis between *ex vivo* baseline WT, injured WT, baseline CD39 TG, and injured CD39 TG mice. N = 4/group

Discussion

Previous research in the laboratory of my advisor, Dr. Richard Gumina, has explored cardioprotection in CD39TG mice with global overexpression of human ectonucleoside triphosphate diphosphohydrolase 1 (CD39) after *in vivo* myocardial infarction. The previous data demonstrated a decrease in infarct size after 20 or 60 minutes of ischemia followed by 24 hours reperfusion in human CD39 TG mice when compared to WT littermates. Furthermore, *in vivo* activation via phosphorylation of Erk 1/2 protein kinase suggested the RISK pathway was one of the mechanisms behind the observed cardioprotection.

The goal of the current work was to determine if global overexpression of human CD39 in *ex vivo* isolated perfusion, without blood and hormonal complications, would confer similar cardioprotection to that previously seen *in vivo*. Furthermore, if cardioprotection was observed in the *ex vivo* Langendorff model, our goal was to determine possible mechanisms behind the cardioprotection.

The results of this study demonstrated that overexpression of human CD39 in the myocardium alone, without the complicating factor of blood, which includes monocytes, immune cells, platelets, and hormones, does indeed protect the heart following *ex vivo* I/R injury. We hypothesized that the mechanism responsible for the observed cardioprotection was due to increased adenosine production. HPLC was used to determine levels of ATP, ADP, AMP, and adenosine following *ex vivo* I/R injury. There was no statistically significant

difference between baseline levels of adenine nucleotides in WT and CD39TG hearts. However, as hypothesized, there was a significant increase in adenosine levels in injured CD39 transgenic hearts when compared to injured wildtype hearts. These results suggest that the mechanism behind the observed cardioprotection does indeed involve increased adenosine generation. The data also showed that there in fact is a decrease in adenosine levels in WT mice after injury, although results were not statistically significant. Adenosine signals through four known receptors: A_1, A_{2a}, A_{2b}, and A_3. Which receptor adenosine signals through depends on cell type, and all adenosine receptors can then activate the RISK pathway. Previous research in Dr. Gumina's lab shows that inhibition of all adenosine receptors with 8-SPT blocked the observed cardioprotection in CD39TG mice. Future studies will look into involvement of the specific adenosine receptors.

Western blot analysis demonstrated increased phosphorylation of key proteins involved in the RISK pathway, Akt, Erk 1/2, and GSK CD39 overexpressing mouse hearts following injury. These results strongly suggest that the RISK pathway plays a role in the observed cardioprotection. Future studies will examine if inhibition of these signaling steps abolishes cardiac protection in CD39 overexpressing hearts.

This project concludes that cardioprotection is observed in human CD39 overexpressing isolated mouse hearts subjected to I/R injury when compared to

wildtype hearts. Furthermore, this work demonstrated that the mechanisms that convey cardioprotection involves increased adenosine generation, as well as activation of the RISK pathway. Further studies are necessary to gain a better understanding of the mechanism behind the cardioprotection before CD39 studies may be applied in a clinical setting. While previous studies *in vivo* showed that global overexpression protects the heart after I/R injury, my study limited overexpression to just the myocytes and vasculature of the heart, and still observed protection. Future studies will examine restricted CD39 overexpression. For example, Dr. Gumina's lab is currently working on mice with myocyte specific overexpression of CD39. Studies with these animals will address if it is myocyte expression that is protective.

The ultimate goal of this and other studies done on CD39 would be to translate findings into a clinical setting and determine if and how CD39 could possibly be used as a therapeutic in organ transplants, stroke, spinal cord injury, and myocardial infarction, which all involve ischemia and reperfusion injury. However, before this work could be applied to humans, researchers must also determine when and how CD39 can be administered to induce organ protection. An important question remaining is if CD39 could be administered during a period of ischemia or after the onset of reperfusion and still provide cardioprotection. While much research remains before clinical application of CD39, these results should indicate that upregulation of CD39 may be an approach to reduce I/R injury.

References Cited

1. Bolli R, Becker L, Gross G, Mentzer R Jr, Balshaw D, Lathrop DA. Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res*. July 23 2004;95 (2): 125-134.

2. Kloner RA, DeBoer LW, Darsee JR, Ingwall JS, Hale S, Tumas J, Braunwald E. Prolonged abnormalities of myocardium salvaged by reperfusion. *Am J Physiol.* Oct 1981;241 (4): H591- 599.

3. Zhu X, Zuo L, Cardounel AJ, Zweier JL, He G. Characterization of *in vivo* tissue redox status, oxygenation, and formation of reactive oxygen species in postischemic myocardium. Antioxid Redox Signal 2007;9:447-55.

4. Xu Y, Huo Y, Toufektsian MC, et al. Activated platelets contribute importantly to myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 2006;290:H692-9.

5. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword?. *J Clin Invest.* 1985.

6. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med.* Sep 13 2007; 357 (11):1121-1135.

7. Wei GZ, Zhou JJ, Wang B, Wu F, Bi H, Wang YM, Yi DH, Yu SQ, Pei JM. Diastolic Ca2+ overload caused by Na+/Ca2+ exchanger during the first minutes of reperfusion results in continued myocardial stunning. *Eur J Pharmacol.* Oct 15 2007; 572 (1): 1-11.

8. Suzuki M, Sasaki N, Miki T, et al. Role of sarcolemmal KATP channels in cardioprotection against ischemia/reperfusion injury in mice. J. Clin. Invest. 2002;109:509-516.

9. Gumina RJ, Pucar D, Bast P, et al. Knockout of Kir6.2 negates ischemic preconditioning-induced protection of myocardial energetics. Am J Physiol Heart Circ Physiol 2003;284:H2106-2113.

10. Zhu X, Liu B, Zhou S, et al. Ischemic preconditioning prevents *in vivo* hyperoxygenation in postischemic myocardium with preservation of mitochondrial oxygen consumption. Am J Physiol Heart Circ Physiol 2007;293:H1442-50.

11. Turko IV, Li L, Aulak KS, Stuehr DJ, Chang JY, Murad F. Protein tyrosine nitration in the mitochondria from diabetic mouse heart. Implications to

dysfunctional mitochondria in diabetes. *J Biol Chem.* 2003 Sep 5;278(36):33972-33977.

12. Hong SJ, Gokulrangan G, Schöneich C. Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry. *Experimental Gerontology.* 2007; 42 (7): 639-651.

13. Kanski J, Behring A, Pelling J, Schöneich C. Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. *Am J Physiol Heart Circ Physiol.* Jan 2005; 288 (1): H371-381.

14. Fryer RM, Auchampach JA, Gross GJ. Therapeutic receptor targets of ischemic preconditioning. Cardiovasc Res 2002;55:520-525.

15. Mei DA, Elliott GT, Gross GJ. KATP channels mediate late preconditioning against infarction produced by monophosphoryl lipid A. *Am J Physiol.* Dec 1996; 271(6 Pt 2): H2723-2729.

16. Kohler D, Eckle T, Faigle M, Grenz A, Mittelbronn M, Laucher S, Hart ML, Robson SC, Muller CE, Eltzschig HK. CD39/ectonucleoside triphosphate diphosphohydrolase 1 provides myocardial protection during cardiac ischemia/reperfusion injury. *Circulation.* Oct 16 2007;116(16):1784- 1794.

17. Peart JN, Headrick JP. Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacol Ther.* May 2007;114(2):208-221.

18. Ninomiya H, Otani H, Lu K, Uchiyama T, Kido M, Imamura H. Complementary role of extracellular ATP and adenosine in ischemic preconditioning in the rat heart. *Am J Physiol Heart Circ Physiol.* May 2002;282(5):H1810-1820.

19. Eckle T, Krahn T, Grenz A, Kohler D, Mittelbronn M, Ledent C, Jacobson MA, Osswald H, Thompson LF, Unertl K, Eltzschig HK. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation.* Mar 27 2007;115(12):1581-1590.

20. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J Am Coll Cardiol.* Jun 7 2005;45(11):1775-1780.

21. Kloner RA, Forman MB, Gibbons RJ, Ross AM, Alexander RW, Stone GW. Impact of time to therapy and reperfusion modality on the efficacy of adenosine in acute myocardial infarction: the AMISTAD-2 trial. *Eur Heart J.* Oct 2006;27(20):2400-2405.

22. Hatakeyama K, Hao H, Imamura T, Ishikawa T, Shibata Y, Fujimura Y, Eto T, Asada Y. Relation of CD39 to plaque instability and thrombus formation in directional atherectomy specimens from patients with stable and unstable angina pectoris. *Am J Cardiol.* Mar 1 2005;95(5):632-635.

23. Brown DA, Lynch JM, Armstrong CJ, et al. Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. J Physiol 2005;564:619-30.

24. Ranki HJ, Budas GR, Crawford RM, Jovanovic A. Gender-specific difference in cardiac ATP- sensitive K(+) channels. J Am Coll Cardiol 2001;38:906-15.

25. Gumina RJ, O'Cochlain DF, Kurtz CE, et al. KATP channel knockout worsens myocardial calcium stress load *in vivo* and impairs recovery in stunned heart. Am J Physiol Heart Circ Physiol 2007;292:H1706-1713.

26. Talukder MA, Kalyanasundaram A, Zuo L, Velayutham M, Nishijima Y, Periasamy M, Zweier JL. Is reduced SERCA2a expression detrimental or beneficial to postischemic cardiac function and injury? Evidence from heterozygous SERCA2a knockout mice. *Am J Physiol Heart Circ Physiol.* Mar 2008; 294 (3): H1426-1434.

27. Headrick, J. P. (1996a). Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol 28*, 1227-1240.